Preparation of Combined Inactivated Vaccine Against Salmonella enteritidis, Salmonella typhimurium and Clostridium perfringens type A and C Toxins

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Abstract: This study was planned to prepare and evaluate the immunizing and protective efficacy of an inactivated combined vaccine containing S. enteritidis, S. typhimurium bacterins and C. perfringens type A and C toxoids. According to the type of adjuvant used, two vaccine formulations were investigated; one of them was adjuvanted with mineral oil and the other with Nigella sativa oil. Three groups of chickens (80 chicks/group) were vaccinated twice S/C. The first group was vaccinated with the mineral oil adjuvanted vaccine, the second group was vaccinated with the N. sativa adjuvanted vaccine and the third group was kept unvaccinated as control group. Blood samples were collected weekly and humeral immune response was measured against Salmonella bacterins using ELISA and against C. perfringens toxoids using SNT. The prepared vaccines induced significantly high protection rates in challenge test using virulent Salmonella strains and significant clearance of S. enteritidis and S. typhimurium from blood and internal organs of the immunized chickens. Also fecal shedding was significantly reduced. The protection level against C. perfringens types A and C was greatly increased. The N. sativa oil adjuvanted formulation stimulated significantly higher protective efficacy as compared with mineral oil adjuvant.

Key words: Inactivated vaccine • S. enteritidis and S. typhimurium • C. perfringens type A • N. sativa • Mineral oil • Adjuvant

INTRODUCTION

The most important food borne zoonotic disease throughout the world is Salmonellosis, as S. enteritidis and S. typhimurium have been responsible for serious hazard to public health [1]. Avian salmonellosis is an inclusive term designating a large group of acute and chronic diseases of poultry caused by one or more member of genus Salmonella. However, particular Salmonella serovars may be encountered more frequently in one country than the other [2, 3]. In Egypt several investigators [4, 5, 6, 7] have isolated many Salmonella serovars particularly, S. typhimurium and S. enteritidis from poultry. Many researchers all over the world have been trying to control and eradicate salmonellosis in poultry by vaccination. Live attenuated salmonella vaccines may be hazardous because the residual virulence due to insufficient attenuation [8] in addition to the possible vertical transmission and interference with diagnosis of field infections in broilers. Inactivated vaccines for the prevention of avian salmonellosis have been reported by several authors [9, 10, 11].

On the other hand, Clostridium perfringens is a leading cause of food-borne poisoning [12] and is the main causative agent of poultry necrotic enteritis (NE), a serious enteric disease of chickens and Turkeys [13] that was first described in Parish [14] and has since been found in all poultry producing countries. NE in chickens manifests itself as an acute or chronic enterotoxaemia [15]. NE is primarily caused by C. perfringens type A and to a lesser extent type C strains [16, 17]. The disease may present itself as an acute clinical or subclinical disease. The clinical symptoms of the acute form are usually very short and often the only signs are sudden increase in mortality. The disease persists in the flock for 5-10 days...
with mortality of 2-50% [18]. Broiler breeder hens vaccinated intramuscularly with candidate vaccines composed of *C. perfringens* type A and type C toxoid adjuvanted with aluminum hydroxide resulted in a strong serum immunoglobulin G response against *C. perfringens* alpha and beta toxins, respectively, in parent hens and specific antibodies were transferred to their progeny [19].

Poultry represents a cheap source of protein throughout the world and important source of transfer food borne bacteria (*S. enteritidis*, *S. typhimurium* and *C. perfringens*) to human, so control of the previous bacteria is a major challenge to the poultry industry. Vaccination is the most effective approach to reduce *Salmonella* contamination inside the commercial poultry farms [21]. So this study came as a response to such need and was planned to prepare and evaluate the immunizing and protective efficacy of an inactivated combined vaccine containing *S. enteritidis*, *S. typhimurium* bacterins and *C. perfringens* type A and C toxoids in chickens. In addition to compare the effect of the two types of adjutants which were used in our study.

**MATERIALS AND METHODS**

**Strains:** *Salmonella typhimurium* strain No (K284/93), *Salmonella enteritidis* strain No (K482/9A), *Clostridium perfringens* type A and type C local isolates were used in this study. These four strains were obtained from the bacterial seed bank of the CLEVB, Abbesia, Cairo, Egypt. These strains are local field isolates recovered and fully identified by the CLEVB and were selected for preparation of a bivalent inactivated vaccine for chickens.

**Vaccines:** The prepared combined vaccine contains the bacterins of *S. enteritidis* and *S. typhimurium* in addition to the toxoid of *Clostridium perfringens* type A (alpha) and *Clostridium perfringens* type C (alpha and beta). The inactivation of *S. enteritidis* and *S. typhimurium* cultures was done using formaldehyde solution 37%, which was added to bacterial suspension in 0.2% final concentration [22]. On the other hand the inactivation of *C. perfringens* type A and C toxins was done using 0.5% formalin and pH was adjusted to 6.0 and kept at 37°C [23].

**Adjuvant:** Two types of adjuvant were compared, where the *Salmonella* bacterins and *C. perfringens* toxoids were emulsified with mineral oil or *Nigella sativa* oil [24, 25] by thoroughly mixing of the aqueous bacterial phase and oil adjuvant phase in ratio of 1:4, the aqueous phase contained 96% inactivated bacterial and toxoid suspension mixed with 4% of tween 80. The *S. enteritidis* and *S. typhimurium* content of the vaccine was adjusted to 10⁷ CFU/0.5 ml vaccine and the toxoid solution (alpha toxoid and beta toxoid) were adjusted to one minimum lethal dose (MLD)/0.5 ml vaccine as a final concentration (determined by mouse intravenous injection test). The oil adjuvant phase was formed from oil adjuvant (mineral oil or *Nigella sativa* oil) and surfactant (span 80) in ratio of 9:1. Finally, thiomersal was added in a concentration of 0.05-0.1g/liter of the vaccine as preservative.

**Quality Control on the Prepared Vaccine:** The sterility, safety and determination of residue of the prepared experimental vaccines (mineral oil and *Nigella sativa* adjuvanted vaccine) were carried out according to the recommendation of the European pharmacopeia [26]. Studying the immune response and duration of immunity of the prepared vaccines was done as described previously [22].

Two weeks old chickens were divided into 3 groups (80 chicks/group). The first group was vaccinated with the mineral oil adjuvanted vaccine and second group was vaccinated with the *N. sativa* oil adjuvanted vaccine, while the third group was kept as control non vaccinated chicks. The chickens in each group were inoculated subcutaneously two times (at 3 week intervals) with 0.5ml of the divalent inactivated mineral oil or *N. sativa* oil adjuvanted vaccine composed of *S. enteritidis*, *S. typhimurium* bacterins and *C. perfringens* type A and C toxoid. The inoculation was made in the middle part of the neck.

**Blood Samples:** Samples were collected before immunization and at one week interval post immunization to measure and evaluate the immune response developed against the immunogenic components of the vaccine. The developed humoral immune response against *S. enteritidis* and *S. typhimurium* was measured using ELISA [27], while that developed against *C. perfringens* type A and C toxins was determined using serum neutralization test as previously described [28].

**Challenge Test:** Challenge was done using 1ml of (MLD) containing 10⁷ CFU or 10⁶ CFU of each of *S. typhimurium* and *S. enteritidis* strains [29, 30]. Challenge was done in two groups; one group was challenged orally by dropper, while the second group was injected intramuscularly in the thigh at three weeks after the injection of vaccine booster doses. Inoculated chickens were observed for one month. The degree of protection was assessed according to the severity of the clinical signs, body weight gain and mortality.
Fecal Samples Collection: It was done before the start of the experiment and after challenge for one month (once/week) using sterile swabs which were inoculated into tetrahionate broth from all chickens including the vaccinated and the control one; all samples were examined bacteriologically in order to detect the level of Salmonella shedding [31].

RESULTS AND DISCUSSION

S. typhimurium, S. enteritidis and C. perfringens could be the cause of serious gastrointestinal diseases as both types of these bacteria can target the intestinal tract, cause death due to the systemic effects of their toxins. It’s reported that the number of Salmonella infected poultry flocks and human beings has been increased substantially in several countries [32].

Necrotic enteritis is occurred when C. perfringens proliferates to high numbers in the small intestine and produces extracellular toxins that damage the intestine [33]. The major toxin believed to be involved is the alpha-toxin which secreted as zinc-metalloenzyme that has both phospholipase C and sphingomyelinase activities, it is the major toxin involved in the pathogenesis of human gas gangrene [34]. So vaccination is the ideal solution to face these problems and overcome the costs and side effects of antibiotics.

The prepared formalin inactivated combined vaccine adjuvanted with mineral oil or N. sativa oil proved to be pure, sterile, safe and free from adverse side effects on chicken productivity and the vaccine residue of formalin was less than 0.05% in both vaccine formulations and the thiomerthal residue was less than 0.02 mg/ml in both vaccine formulations. These values are within the permissible limits [35].

In the mineral oil adjuvanted inactivated combined vaccine the ELISA antibodies titers against S. enteritidis and S. typhimurium reached to 690.1 and 715.3 after the 3rd week from primary vaccination, respectively and to 2587.6 and 2133.1 after 3rd week from the booster dose, respectively as showed in (Figure 1 & 2). Similar results about the enhancement of anti-Salmonella antibody production by the use of mineral oil adjuvants Salmonella vaccines have been reported by several authors [36, 11, 37] also these results were in harmony with that of Hassan et al. [31] who used mineral oil adjuvant vaccines to compare local vaccine of S. typhimurium and S. enteritidis with the imported one, as local vaccine induced high protection rates in challenge test with reduced fecal shedding and higher antibody response.

The seed of N. sativa has been reported to have many biological properties including antibacterial effect [38]. The oil fraction of N. sativa contains thymoquinone, which has immuno-potentiating activities as well as antioxidative effect [39]. In N. sativa oil adjuvanted vaccinethel ELISA antibody titer against S. enteritidis and S. typhimurium reached to 601.1 and 613.4, respectively, after the 3rd week from primary immunization and to 1701.8 and 1210.8 respectively, after the 3rd week post the boosting dose (Figure 1 & 2). Significant difference in antibody enhancements effect of both adjuvants was recorded, where N. sativa oil adjuvanted vaccine stimulated lower antibody titer against Salmonella strains as compared with the mineral oil adjuvanted one.

In evaluating the protective value of both adjuvanted vaccine formulations, challenge test was performed as previously described [30]. This test is considered the master test for determination of the protective value of a vaccine [40].

In the mineral oil adjuvanted vaccine the protective value against virulent Salmonella strain reached to 62.5% and 60% after oral or I/M challenge, respectively, as compared with protection rate of 71.8% and 70% post oral or I/M challenge in chickens vaccinated with N. Sativa oil adjuvanted vaccine as explained in (Table 1). The achieved protection rates by both vaccine formulations are accepted to pass the vaccine for use according to that mentioned by Heddleston[41] and the recommendation of the Egyptian veterinary codex, CLEVB [35].

The results of fecal shedding in the group of chicken vaccinated with the mineral oil adjuvanted vaccine demonstrate that Salmonella strains could be re-isolated at the rates of 23%, 10.7% and 0.07% in the 1st, 2nd and 3rd week after oral challenge with virulent Salmonella strains, respectively, while After intramuscular challenge, Salmonella strains could be re-isolated at rates of (13.3%), (28.5%) and (14.2%) in the 1st, 2nd and 3rd weeks post challenge, respectively. on the other hand the results of fecal shedding in the group of chicken vaccinated with the N. sativoadjuvanted vaccine revealed that Salmonella strains could be re-isolated from the vaccinated chickens at the rates of (32%), (0.9%) and (0.09%) in the 1st, 2nd and 3rd weeks, respectively, after oral challenge with virulent Salmonella strains, while Salmonella strains re-isolated at rates of (13.3%), (21.4%) and (10.7%) in the 1st, 2nd and 3rd weeks, respectively, after intramuscular challenge with virulent Salmonella strains. Regarding the control non-vaccinated birds the re-isolation rates were (79%) (81%), (62.5%) and (25%) at the 1st, 2nd, 3rd and 4th weeks post oral challenge, respectively. Following challenge by
Table 1: Protective efficacy of formalized mineral oil and Nigella sativa oil adjuvanted bivalent vaccine in vaccinated chicken after challenge with virulent S. enteritidis and S. typhimurium strains

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Rout of challenge</th>
<th>Type of Vaccine</th>
<th>Total No. of birds</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
<th>Dead</th>
<th>Total</th>
<th>Mortality rate (%)</th>
<th>Protection%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral oil adjuvanted vaccine</td>
<td>Orally</td>
<td>Adjuvant vaccine group</td>
<td>40</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>12/40</td>
<td>30%</td>
<td>62.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non vaccinated group</td>
<td>40</td>
<td>11</td>
<td>13</td>
<td>8</td>
<td>0</td>
<td>32/40</td>
<td>80%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra muscular</td>
<td>Adjuvant vaccine group</td>
<td>40</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>16/40</td>
<td>30%</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non vaccinated group</td>
<td>40</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td>0</td>
<td>40/40</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N. sativa adjuvanted vaccine</td>
<td>Orally</td>
<td>Adjuvant vaccine group</td>
<td>40</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>9/40</td>
<td>22.5%</td>
<td>71.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non vaccinated group</td>
<td>40</td>
<td>11</td>
<td>13</td>
<td>8</td>
<td>0</td>
<td>32/40</td>
<td>80%</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Intra muscular</td>
<td>Adjuvant vaccine group</td>
<td>40</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>12/40</td>
<td>30%</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Non vaccinated group</td>
<td>40</td>
<td>20</td>
<td>15</td>
<td>5</td>
<td>0</td>
<td>40/40</td>
<td>100%</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Feecal shedding of Salmonella spp. after challenge of chicken vaccinated with inactivated bivalent mineral oil adjuvanted vaccine

<table>
<thead>
<tr>
<th>Rout of challenge</th>
<th>Type of vaccine</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally</td>
<td>Vaccinated group</td>
<td>7/30(23%)</td>
<td>3/28(10.7%)</td>
<td>2/28(0.07%)</td>
<td>0/28(0%)</td>
</tr>
<tr>
<td></td>
<td>Control non vaccinated group</td>
<td>23/29(79%)</td>
<td>13/16(81.2%)</td>
<td>5/8(62.5%)</td>
<td>2/8(25%)</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>Vaccinated group</td>
<td>3/26(13.3%)</td>
<td>8/24(28.5%)</td>
<td>4/24(16.6%)</td>
<td>0/24(0%)</td>
</tr>
<tr>
<td></td>
<td>Control non vaccinated group</td>
<td>10/20(50%)</td>
<td>12/15(80%)</td>
<td>3/5(60%)</td>
<td>All died</td>
</tr>
</tbody>
</table>

Table 3: Feecal shedding of Salmonella spp. after challenge of chicken vaccinated with inactivated bivalent Nigella sativa oil adjuvant vaccine

<table>
<thead>
<tr>
<th>Rout of challenge</th>
<th>Type of vaccine</th>
<th>1st week</th>
<th>2nd week</th>
<th>3rd week</th>
<th>4th week</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orally</td>
<td>Vaccinated group</td>
<td>10/32(31.2%)</td>
<td>3/31(9%)</td>
<td>3/31(9.9%)</td>
<td>0/31(0%)</td>
</tr>
<tr>
<td></td>
<td>Control non vaccinated group</td>
<td>23/29(79%)</td>
<td>13/16(81.2%)</td>
<td>5/8(62.5%)</td>
<td>2/8(25.5%)</td>
</tr>
<tr>
<td>Intramuscular</td>
<td>Vaccinated group</td>
<td>4/30(13.3%)</td>
<td>6/28(21.4%)</td>
<td>3/28(10.7%)</td>
<td>0/28(0%)</td>
</tr>
<tr>
<td></td>
<td>Control non vaccinated group</td>
<td>10/20(50%)</td>
<td>12/15(80%)</td>
<td>3/5(60%)</td>
<td>All died</td>
</tr>
</tbody>
</table>
intramuscular route the re-isolation rates were (50%), (60%) and (80%) at the 1st, 2nd and 3rd weeks post challenge (Tables 2 & 3), these results were closely related to that previously reported [42, 36] as the authors in these 2 studies had similar re-isolation rates from the fecal samples collected from immunized chickens.

The analysis of the immune responses developed against C. perfringens type A and C toxoids showed that the mineral oil adjuvant enhanced the antibody production against C. perfringens type A and C toxins as measured by SNT, which reached to 0.9 and 5 IU/ml, respectively after 3rd week post primary immunization and reached to 2.6 and 8.8 IU/ml after 3 weeks post boosting, this result agrees with that published previously [43]. In chickens immunized with the N. sativa oil adjuvanted inactivated combined vaccine, the SNT measured antibody titers against C. perfringens type A and C toxins reached to 1.5 and 6 IU/ml after 3rd week post primary immunization and 3.1 and > 10 IU/ml after the 3rd week of boosting dose. These results showed that N. sativa enhanced the antibody production against the toxins more strongly than the mineral oil adjuvant, this agrees with that obtained in previous reports [44, 45]. The European pharmacopeia reported that; the protective antibody titer against the Type A and Type C toxins of C. perfringens is 0.5 IU/ml for type A toxin and 8 IU/ml for the type C toxin [26], this indicated that the prepared two vaccine formulations in this study were capable of achieving this protective levels of antibodies against Type C toxins only after the second booster dose.

The difference of the effect of N. sativa on the level of the produced antibodies according to the type of the immunizing agent (lower antibody titer in case of Salmonella antigens and higher antibody levels in case of C. perfringens toxoids) is difficult to be explained. It might be attributed to the nature of the antigen where it was suspended intact bacteria in case of Salmonella compared to soluble protein in case of the used toxoids. Also the chemical composition may play a role. All these points and others need more investigations.

REFERENCES


